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Interactive Regulation of *Azorhizobium nifA* Transcription via Overlapping Promoters

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The *Azorhizobium nifA* promoter (P*nifA*) is positively regulated by two physiological signal transduction pathways, NtrBC, which signals anabolic N status, and FixLJK, which signals prevailing O_2 status. Yet, P*nifA* response (gene product per unit time) to these two activating signals together is more than twice that of the summed, individual signals. In the absence of NIFA, a negative P*nifA* autoregulator, the fully induced P*nifA* response is more than 10-fold greater than that of summed, individual signals. Given this synergism, these two signal transduction pathways must interactively regulate P*nifA* activity. P*nifA* carries three *cis*-acting elements, an anaerobox, which presumably binds FIXK, a NIFAbox, which presumably binds NIFA itself, and a σ^{54}box, which presumably binds σ^{54} initiator, a subunit of RNA polymerase. For combinatorial analysis, single, double, and triple promoter mutations were constructed in these *cis*-acting elements, and P*nifA* activities were measured in six different *trans*-acting backgrounds, i.e., *fixK*, *fixJ*, *nifA*, *ntrC*, *rpoF*, and wild type. Under all physiological conditions studied, high-level P*nifA* activity required both FIXK in *trans* and the anaerobox element in *cis*. Surprisingly, because P*nifA* was hyperactive with a mutated σ^{54}box, this *cis*-acting element mediates both negative and positive control. Because P*nifA* hyperactivity also required a wild-type upstream NIFAbox element, even in the absence of NIFA, a second upstream *nifA* transcription start superimposed on the NIFAbox element was hypothesized. When *nifA* mRNA 5′ start points were mapped by primer extension, both a minor upstream transcript(s) starting 45 bp distal to the anaerobox and a major downstream transcript starting 10 bp distal to the σ^{54}box were observed. In *Azorhizobium*, RNA polymerase σ^{54} initiator subunits are encoded by a multigene family, which includes *rpoF* and *rpoN* genes. Because *rpoF* mutants show an Ntr^+ phenotype, whereas *rpoN* mutants are Ntr^-, multiple σ^{54} initiators are functionally distinct. Two independent *rpoF* mutants both show a tight Nif^- phenotype. Moreover, *rpoF* product σ^{54}F is absolutely required for high-level P*nifA* activity. In summary, the *Azorhizobium nifA* gene carries overlapping housekeeping-type and σ^{54}-type promoters which interactively respond to different signals. Effectively, the upstream, housekeeping-type promoter responds to FIXK and positively regulates the downstream, σ^{54}-type promoter. The downstream, σ^{54}-type promoter responds to NTRC and negatively regulates the upstream, housekeeping-type promoter. In terms of transcript yield, the upstream, housekeeping-type promoter is therefore weak, and the downstream, σ^{54}-type promoter is strong.

Unique among characterized bacteria, *Azorhizobium caulinodans* fixes N_2 at high rates both in free-living culture and in symbiosis with *Sesbania* spp. host legumes (17). Both structural and regulatory (*nif*) genes for N_2 fixation are strongly conserved across the *Bacteria*. Among the latter is the *nifA* gene, which encodes a master transcriptional activator. While *nifA* coding sequences are highly conserved, *nifA* expression patterns are quite different, even among related N_2-fixing bacteria. Different *nifA* regulatory circuits reflect orthologous *cis*- and *trans*-acting elements "wired" together quite differently (21). *Klebsiella pneumoniae*, a microaerophilic diazotroph, regulates *nifA* expression hierarchically through two signal transduction pathways. The primary pathway, low anabolic N signal → NTRB → NTRC → *nifLA*, allows NIFA synthesis, while a secondary pathway, high O_2 signal → NIFL ⟞ NIFA → *nifLA*, modulates NIFA levels (⟞, negative regulation; →, positive regulation). In *K. pneumoniae*, both NTRB→NTRC and NIFL ⟞ NIFA constitute cognate pairs of environmental sensor and response regulator. The NTRB→NTRC pair, when stimulated by anabolic N limitation, activates transcription at specific targets, among them the *nifLA* operon (18). In an amplification cascade, *K. pneumoniae* NIFA so produced not only activates transcription at other *nif* operons, it also autoregulates its own transcription (7). NIFA negatively responds to its cognate environmental sensor, NIFL, which somehow monitors endogenous O_2, and to high anabolic N levels (26, 36). Both NIFA and NTRC specifically bind DNA and activate RNA polymerase σ^{54}-type transcription initiation complexes. In *K. pneumoniae*, promoter targets carry distinct upstream activating elements which bind either NIFA or NTRC (8, 47) as well as diagnostic −12 and −24 elements, which bind σ^{54} initiator either alone or complexed with core RNA polymerase (5, 6). This integration of *cis*- and *trans*-acting elements allows global transcriptional control of targeted operons.

In contrast, *Rhizobium meliloti nif* genes are regulated by physiological O_2 but not by anabolic N levels. In *R. meliloti* cultures, since *nif* gene products are only very weakly expressed and N_2 fixation is not observed, the *nif* genes probably respond to as-yet-uncharacterized symbiotic signals. While various *Rhizobium* species all carry a highly conserved NTRB→NTRC cognate pair, its targets do not include *nif* genes (57). While *R. meliloti* and *K. pneumoniae* NIFA are orthologous, no *Rhizobium* NIFA cognate environmental sensor (NIFL ortholog) activity has been identified. Instead, in *R. meliloti*, a limiting O_2 signal activates FIXL→FIXJ, yet another cognate environmental sensor and response regulator pair. FIXL, a transmembrane hemoprotein sensor, phosphorylates cytosolic FIXJ, its response regulator, which activates both *fixK* and *nifA* transcription (4, 10, 12). As with *K. pneumoniae*, *R. meliloti* NIFA

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then *trans*-activates constituent *nif* operons during symbiosis. FIXK *trans*-activates genes expressed during microaerobiosis both in culture and in symbiosis. *R. meliloti nif* promoters also carry homologous −12 and −24 sequences which serve as targets for RNA polymerase σ^{54} (38) initiation complexes.

Azorhizobium nif genes, although dispersed, also constitute a NIFA regulon (15, 41). As with all rhizobia, *Azorhizobium* FIXL, FIXJ, and FIXK together regulate *nifA* transcription initiation (27). However, *Azorhizobium* FIXK activates whereas *R. meliloti* FIXK nominally represses *nifA* expression (28). Although its *nif* genes respond both to FIXL→FIXJ→FIXK and to NTRB→NTRC stimulation in culture, *Azorhizobium* shows atypical Ntr regulation. While *Azorhizobium ntrC* mutants both fix N_2 in culture poorly and form nodules slowly, these nodules fix N_2 at almost wild-type rates (43). In addition, a third sensor-regulator couple, NTRY→NTRX, also somehow modulates *Azorhizobium nif* gene expression (42). From these results, we hypothesized that the *Azorhizobium nifA* gene might show unusual regulation, including properties of both *K. pneumoniae* and *R. meliloti.*

As detailed here, *Azorhizobium* P*nifA* is regulated by three *trans*-activators, FIXK, NTRC, and NIFA itself, and requires σ^{54}F, a specific σ^{54} initiator. From sequence comparisons, P*nifA* carries three *cis*-acting elements, inferred DNA-binding sites for FIXK, NIFA, and σ^{54}-type initiators, which are tightly clustered. Overlapping the NIFA site is a σ^{70}-type promoter for housekeeping-type genes. For combinatorial analysis, *Azorhizobium* strains carrying mutations in these three discrete, *cis*-acting P*nifA* elements were constructed in six *trans*-acting backgrounds, namely, *fixK*, *fixJ*, *nifA*, *ntrC*, *rpoF*, and wild type. In summary, these *cis*- and *trans*-acting elements constitute two overlapping promoters, which interactively regulate P*nifA* activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains, plasmids, and bacteriophages employed are listed in Table 1. *Azorhizobium* strains were grown in either GYPCS or BASAL medium (9, 14). Two *rpoF* mutants, strains 60002L and 60064R, were tested for secondary N-dependent growth on solidified BASAL medium containing succinate (0.4%) as a C source and either nitrate, L-valine, or L-leucine (10 mM) as the sole N source. To test growth on dicarboxylic acids, BASAL medium contained ammonium sulfate (20 mM) as the sole N source and either succinate, L-malate, or fumarate (0.4% [wt/vol]) as a C source.

Molecular cloning and mutagenesis. For site-directed mutagenesis, the *Azorhizobium nifA* promoter region (P*nifA*) was cloned in pBluescript SK^+ as part of a 415-bp *Xho*I-*Eco*RV fragment. The resulting plasmid, pVNA14, was transformed into *E. coli* CJ236 for synthesis of uracil-laden, single-stranded DNA (31). The P*nifA* FIXK binding site (anaerobox), NIFA binding site (NIFAbox), and σ^{54} binding site (σ^{54}box) regions were mutagenized in all possible combinations as described previously (20) with the following synthetic oligonucleotides:

5′-dGGATACCTTCAAAATGGGATCCAGATCAAAGC-3′ (TTGA = wild-type anaerobox)
5′-dCGGTTTCCATTATAGTAATTCCATTAAATCG-3′ (AACA = wild-type NIFAbox)
5′-dAACACCGACAGGATTCTGATCCCTCGCAGC-3′ (AGGC = wild-type σ^{54}box)

All mutations were verified by dideoxynucleotide chain termination sequencing of the resulting pVNA14 derivatives. An *Eco*RV-*Eco*RI fragment carrying a promoterless *Escherichia coli uidA* DNA cassette from pKSG1 was cloned into similarly digested, mutagenized pVNA14 derivatives. These plasmids were linearized with *Bam*HI or *Xba*I, treated with *E. coli* DNA polymerase I Klenow fragment, and redigested with *Xho*I. The resulting 2.3-kbp P*nifA*::*uidA*-containing DNA fragments were purified electrophoretically in low-melting-point agarose, recloned into *Xho*I-linearized pVSN1500 that had been partially digested with *Eco*RV, and similarly purified. The resulting plasmids (pVSN30xx series) carried *uidA* transcriptional fusions in the complete *Azorhizobium* 4.9-kbp *Eco*RI fragment carrying *nifA*. These *uidA* fusions served as P*nifA* reporters for the eight *nifA* promoter variants (see Fig. 1 and 2) that were then introduced at the *Azorhizobium nifA* locus by triparental conjugation from *E. coli*/pVSN30xx donors by employing pRK2073 as the conjugation-proficient helper in *trans* (13). All *Azorhizobium* tetracycline-resistant transconjugants proved to be partial merodiploids for the *nifA* locus and thus resulted from cointegration of pVSN30xxx plasmids. From these *nifA* partial merodiploids, *nifA*::*uidA* haploid derivatives carrying perfect gene replacements were identified by screening for Tc^s descendants, as described previously (9). For each genotype subsequently tested, two independent mutants were analyzed.

The *Azorhizobium rpoF* locus was physically isolated from mutants 60064 and 60002 by Vi cloning as described previously (16). Recombinant phages λNif144, λNif15, and λNif65 isolated from an *Azorhizobium* wild-type genomic library (15) were probed with pRJ7734 and pRJ7693 carrying the *Bradyrhizobium japonicum* $rpoN_1$ and $rpoN_2$ genes (30). A 13.2-kbp *Sal*I fragment from λNif15 was subcloned into pSUP2024aa, partially digested with *Sal*I, yielding pVSN4000, which was then introduced into *Azorhizobium* strains as described above. Nif phenotypic characterizations and complementation tests were carried out by acetylene reduction assays and by N_2-dependent growth tests on defined solid media as described before (16).

Physiological *nifA* induction and β-Gus activity assays. *Azorhizobium* gene fusion derivatives were inoculated in NIF medium (15) supplemented with 0.4% glutamine as the N source and cultured to the late exponential phase. The cells were washed and diluted (to an A_{600} of 0.40) in ice-cold NIF medium containing 100 μM nicotinate. Aliquots (15 ml) were transferred to 125-ml flasks that were then stoppered, sparged for 5 min with an optimal gas mixture (96% argon, 1% CO_2, and 3% O_2), and shaken for 6 h at 30°C. To achieve anabolic N excess, 0.4% (wt/vol) L-glutamine was added to selected samples prior to induction; to achieve O_2 excess, other samples were cultured unstoppered. β-Glucuronidase (β-Gus) activities were measured by a fluorometric assay (25); total protein concentrations were determined with a Folin phenol reagent assay (44). All induction experiments were conducted in triplicate and repeated until the standard error in β-Gus activities was below 15%.

Transcription start point mapping. Representative *Azorhizobium* strains were precultured in NIF medium containing excess N (0.4% L-glutamine) and 100 μM nicotinate. Early-exponential-phase cultures were pelleted by centrifugation, washed twice with 2× P (phosphate) buffer (16) resuspended in NIF medium (15) containing 100 μM nicotinate, and sparged for 8 h at 28°C with 3% O_2, 1% CO_2, balance N_2. Total RNA was prepared from induced cultures by guanidium thiocyanate lysis followed by sedimentation through a cushion of CsCl solution (1.80 g ml^{-1}) (51). Total RNA was effectively purified from otherwise abundant acidic polysaccharide, which banded in the CsCl solution. The pelleted RNA was dissolved in Tris-EDTA buffer containing 0.1% sodium dodecyl sulfate (SDS), adjusted to pH 5.2 with 3 M sodium acetate, and precipitated with cold ethanol (51). To map 5′ start points for *nifA* mRNAs, a synthetic deoxynucleotide primer (3′-AGCTCGTGGCGGCCTCTGTA-5′) distal to the *nifA* translation start (Fig. 1) was radiolabeled with polynucleotide kinase in the presence of [γ-^{32}P]ATP. Purified, total RNA was resuspended and annealed with the radiolabeled primer. The four deoxynucleoside triphosphates were added, and the mixture was incubated with purified retroviral reverse transcriptase (Superscript; Bethesda Research Laboratories) lacking RNase H activity, all as described in the vendor's protocol. The same synthetic deoxynucleotide was used as a primer in standard dideoxynucleotide chain termination DNA sequencing reactions with the 4.9-kbp *Eco*RI (*nifA*$^+$) DNA fragment as a template. Total RNA primer extension reaction mixtures and DNA sequencing reaction mixtures were loaded on adjacent lanes of standard polyacrylamide gels for DNA sequencing, subjected to denaturing electrophoresis as for DNA sequencing, and analyzed by autoradiography.

RESULTS

***Azorhizobium* P*nifA* is interactively regulated in response to limiting O_2 and limiting N physiological signals.** In previous work, the *Azorhizobium nifA* promoter region (P*nifA*) was sequenced and scanned for putative *cis*-acting elements. In proximal-to-distal physical order, canonical FIXK binding site (anaerobox), consensus NIFA binding site (NIFAbox), and σ^{54}-dependent, consensus −12 and −24 transcription start point (σ^{54}box) elements were identified (40) (Fig. 1). In further experiments designed to study these P*nifA* elements in detail, we constructed allelic (genomic) *nifA* reporters. With the recombinant plasmid pKSG1 used as the substrate (Table 1), a complete P*nifA* element was spliced in vitro upstream of the *E. coli uidA* open reading frame for β-Gus and then recombined into target plasmid pVSN1500 (Table 1), yielding *nifA*::*uidA* transcriptional fusions (see Materials and Methods). Combinatorial dinucleotide changes in each of the three inferred P*nifA* elements were then introduced in *cis* to the reporter, and mutant P*nifA* promoter constructs were verified by DNA sequencing (see Materials and Methods). Resulting P*nifA*::*uidA* plasmids were then mobilized from *E. coli* to the

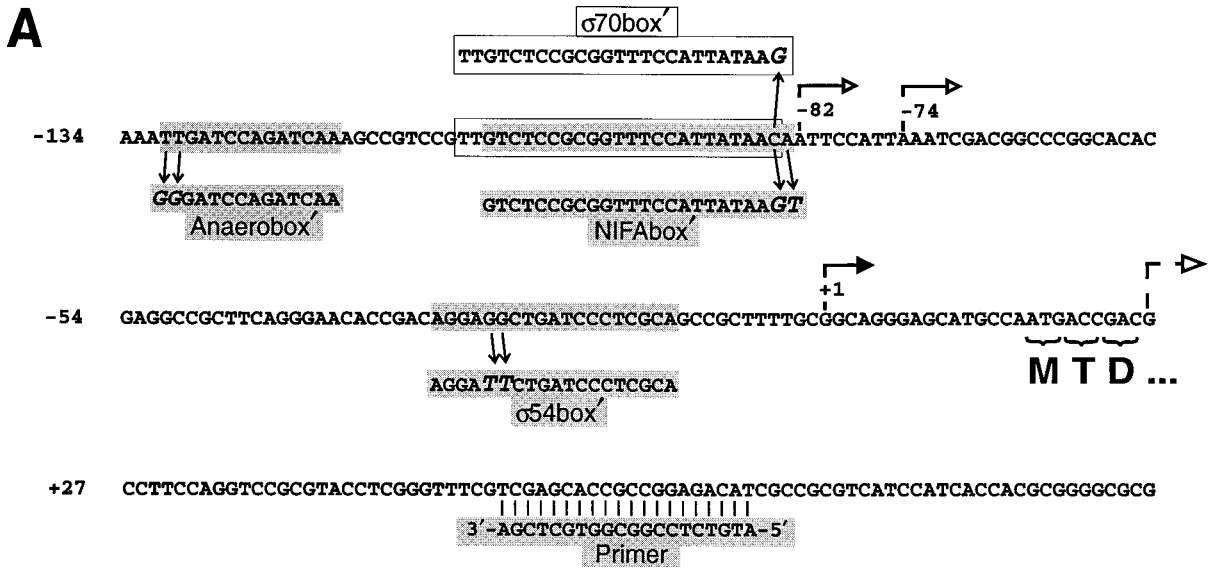
TABLE 1. Bacterial strains and plasmids

Strain, plasmid, or phage	Genotype or phenotype	Source or reference
E. coli		
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	H. W. Boyer
DH5α	*supE44 hsdR17 recA1 endA1 thi-1 gyrA96 relA1* ΔU169 (ϕ80 *lacZ*ΔM15)	Life Technologies, Inc.
CJ236	*dut-1 ung-1 thi relA1*/pCJ105 (F′ *cat*$^+$) Cm^r	31
XL-1 Blue	*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1* Δ(*lac-proAB*) [F′ *lacI*^{q} *proA*$^+$*B*$^+$ *lacZ*ΔM15 Tn*10*] Tc^r	W. O. Bullock
A. caulinodans		
57100	Wild type	
60107	*nifA*::VP2021 Tc^r Km^r Sm^r	15
60034	*nifA*::VP2021 Tc^r Km^r Sm^r	15
60107R	60107 *nifA*::IS*50*R	
60034R	60034 *nifA*::IS*50*R	
57601	*fixK1*::*npt* Km^r	28
57592	*fixJ2*::*npt* Km^r	27
571C6	*ntrC*::Tn*5* Km^r	43
60002	*rpoF*::pVP2021 Tc^r Km^r Sm^r	
60002L	60002 *rpoF*::IS*50*L	
60064	*rpoF*::pVP2021 Tc^r Km^r Sm^r	
60064R	60064 *rpoF*::IS*50*R	
60301	P*nifA*::*uidA nifA*$^+$	
60302	60002L *rpoF2*L P*nifA*::*uidA nifA*$^+$	
60312	60301 P*nifA*::*uidA* (haploid)	
60341	60034R P*nifA*::*uidA nifA34*::IS*50*R	
60361	57601 *fixK* P*nifA*::*uidA nifA*$^+$	
60364	60064L *rpoF64*R P*nifA*::*uidA nifA*$^+$	
61071	60107R P*nifA*::*uidA nifA107*::IS*50*R	
Plasmids		
pSUP202	pBR325 *mob* Ap^r Tc^r Cm^r	54
pSUP2024AA	pSUP202 *Eco*RI-*Bam*HI-*Sal*I-*Pst*I linker Ap^r Tc^r Cm^r	
pVSN4000	pSUP2024AA *rpoF*$^+$ (13.2 kbp) Ap^r Tc^r	
pVSN1500	pSUP202 *nifA*$^+$ (4.9 kbp) Ap^r Tc^r	
pVSN3001	pVSN1500 P*nifA*$^+$::*uidA* Ap^r Tc^r	
pVSN3010	pVSN1500 P*nifA*::*uidA*, anaerobox TT→GG, Ap^r Tc^r	
pVSN3020	pVSN1500 P*nifA*::*uidA*, NIFAbox AC→GT, Ap^r Tc^r	
pVSN3030	pVSN1500 P*nifA*::*uidA*, σ^{54}box GG→TT, Ap^r Tc^r	
pVSN3040	pVSN1500 P*nifA*::*uidA*, NIFAbox AC→GT, σ^{54}box GG→TT, Ap^r Tc^r	
pVSN3050	pVSN1500 P*nifA*::*uidA*, anaerobox TT→GG, NIFAbox AC→GT, Ap^r Tc^r	
pVSN3060	pVSN1500 P*nifA*::*uidA*, anaerobox TT→GG σ^{54}box GG→TT, Ap^r Tc^r	
pVSN3070	pVSN1500 P*nifA*::*uidA*, anaerobox TT→GG, NIFAbox AC→GT, σ^{54}box GG→TT, Ap^r Tc^r	
pRK2073	ColE1 RK2 Tra$^+$ Sp^r	13
pBluescript	f1 *ori* Ap^r	Stratagene, Inc.
pRJ7734	pBluescript II KS$^+$ *B. japonicum rpoN*$_{2^+}$ (3.0 kbp *Eco*RI-*Cla*I fragment) Ap^r	31
pRJ7693	pBluescript II KS$^-$ *B. japonicum rpoN*$_{1^+}$ (1.7 kbp *Eco*RI-*Hin*dIII fragment) Ap^r	31
pKSG1	pBluescript SK$^+$, *Pst*I *uidA′* cassette from λTn*5*::*uidA* Ap^r	
Bacteriophages		
λEMBL3	λ*sbhI* λ b189 (*int-29 ninL44 trpE*) KHσ^{54} *chiC* srI λ4 *nin-5 srI* λ5	22
λNif15	λEMBL3, *Azorhizobium rpoF* locus	
λNif65	λEMBL3, *Azorhizobium rpoF* locus	
λNif144	λEMBL3, *Azorhizobium rpoF* locus	
λTn*5*::*uidA1*	*uidA*$^+$ Tc^r Km^r	53

Azorhizobium wild type and to *nifA*, *fixJ*, *fixK*, *ntrC*, and *rpoF* mutants by bacterial conjugations. Because they lacked the capacity to replicate in *Azorhizobium* strains yet carried a selectable tetracycline resistance gene, these recombinant ColE1 replicons constituted *Azorhizobium* suicide vectors. *Azorhizobium* transconjugants were selected as stable Tc^r derivatives which, when analyzed in detail, were verified as *nifA* merodiploids. These strains invariably carried true genome::plasmid cointegrates, which presumably arose from homologous, single-recombination events.

As verified by physical mapping experiments, *Azorhizobium nifA* merodiploids carried both donor P*nifA*::*uidA* and recipient *nifA* alleles at this locus (Fig. 2). Upon cointegration, as the linkage between mutated P*nifA* elements and their β-Gus reporters was preserved, no crossover events in promoter sequences had occurred. Indeed, because introduced P*nifA* mutations were, at most, 316 bp upstream from the reporter open reading frame and because the homologous DNA template extended some 4.9 kbp, most merodiploids ought to have arisen from single-crossover events elsewhere at this locus. For each desired genotype, two independent cointegrants were obtained and analyzed. In all cases, the two isolates were phenotypically and physically indistinguishable. As subsequently determined (discussed below), the three combinatorial P*nifA* mutations yielded characteristic β-Gus induction patterns in all strains tested. Among all isolated cointegrants, no wild-type P*nifA* elements fused to β-Gus reporters were identified.

To first test the consequences of adding both additional P*nifA* and linked donor plasmid sequences at the *Azorhizobium nifA* locus, β-Gus activities were compared in P*nifA*$^+$::*uidA*



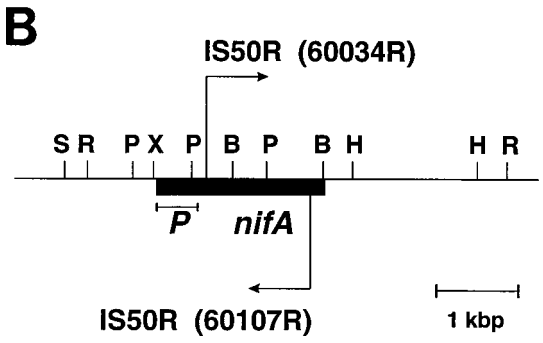


FIG. 1. (A) The *Azorhizobium nifA* promoter region. Four *cis*-acting elements, i.e., anaerobox, NIFAbox, σ^{70}box, and σ^{54}box sequences, are boxed. Dinucleotide changes introduced by in vitro site-directed mutagenesis at each *cis*-acting element are italicized. The first three translated codons are bracketed; for simplicity, the subsequent coding sequence (40) is not displayed. The synthetic oligodeoxynucleotide used for primer extension transcription start point mapping experiments (primer) is also displayed. Mapped transcription start points are diagrammed as arrows: the σ^{54}-dependent start point maps to position +1; the σ^{54}-independent start point maps to position −74. (B) Physical map of the *nifA* locus. IS*50*R insertion points giving rise to mutants 60034R and 60107R (Table 1) are indicated. Abbreviations: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; X, *Xho*I.

nifA::IS*50*R merodiploid and P*nifA*$^+$::*uidA* haploid derivatives. To obtain the latter, P*nifA*$^+$::*uidA nifA*$^+$ merodiploids were cultured nonselectively for 20 bacterial generations and then plated; colonies were screened for Tc^s, Nif$^-$, and Gus$^+$ phenotypes. By physical mapping, these isolates had indeed lost all plasmid sequences as well as parental *nifA*$^+$ alleles and thus represented *nifA* haploid strains carrying perfect gene replacements (Fig. 3). Two independent haploid P*nifA*$^+$::*uidA* derivatives, strains 60312 and 60314, exhibited β-Gus induction profiles mirroring that of P*nifA*$^+$::*uidA nifA*::IS*50*R merodiploid strain 60341 (discussed below). Therefore, when tested for P*nifA* reporter activities, P*nifA* merodiploids and P*nifA* haploids behaved similarly.

When P*nifA*$^+$::*uidA nifA*$^+$ merodiploid strain 60301 was cultured in N excess under air, P*nifA* activities were 13% of fully induced levels (Table 2). Partial P*nifA* induction in response to either O_2 limitation (23% fully induced) or N limitation (24% fully induced) was observed. Under simultaneous N limitation and O_2 limitation, P*nifA* was fully induced. However, the P*nifA* response to simultaneous limiting N and limiting O_2 signals was more than twice the sum of the partial inductions (Table 2). Therefore, N limitation and O_2 limitation signal transduction pathways interactively regulate P*nifA* activity.

***Azorhizobium* NIFA regulates its own transcription both positively and negatively.** *Azorhizobium* NIFA regulates its own transcription (40, 46). To study autoregulation in more detail, the following three P*nifA*$^+$::*uidA* reporter strains were compared: (i) P*nifA*::*uidA* haploid strain 60314, (ii) P*nifA*::*uidA nifA34*::IS*50*R (*nifA34*R allele) merodiploid strain 60341, in which IS*50*R is P*nifA* proximal, and (iii) P*nifA*::*uidA nifA107*::IS*50*R (*nifA107*R allele) merodiploid strain 61071, in which IS*50*R is P*nifA* distal (Fig. 1 to 3). Both strains 60314 and 60341 lacked detectable NIFA activity. For both strains, under simultaneously O_2-limited and N-limited culture, P*nifA* was hyperactivated (129 and 157% of wild-type induced levels). Therefore, NIFA represses its own transcription.

In both P*nifA*::*uidA* haploid strain 60314 and P*nifA*::*uidA nifA34*R merodiploid strain 60341, P*nifA* induction in response to physiological N limitation alone was almost completely abolished; β-Gus activities were reduced to levels (5 to 7% of the wild-type induced level) even lower than those observed in uninduced cultures of P*nifA* merodiploids carrying a wild-type *nifA*$^+$ allele. Therefore, NIFA positively regulates its own transcription in response to physiological N limitation. Although P*nifA* induction in response to O_2 limitation alone was also poor (9 to 12% of the wild-type induced level), relative induction above basal levels was similar to that of merodiploids carrying a wild-type *nifA*$^+$ allele. Interestingly, in strain 61071, which may express C-terminally truncated $NIFA_{107R}$ (Fig. 1), partial P*nifA* induction in response to either O_2 limitation or N limitation was unaffected (27 or 23% of the wild-type induced level, respectively), but negative autoregulation was abolished (133% of the wild-type induced level). Therefore, the C-truncated $NIFA_{107R}$ product might retain some *trans*-activating capabilities but might not bind DNA.

At P*nifA*, FIXK is a strong and NTRC is a weak *trans*-activator, yet both are required for an integrated, high-level response. To study potential *trans*-activators, P*nifA*::*uidA nifA*$^+$ merodiploids were also constructed in *fixK* strain 57601,

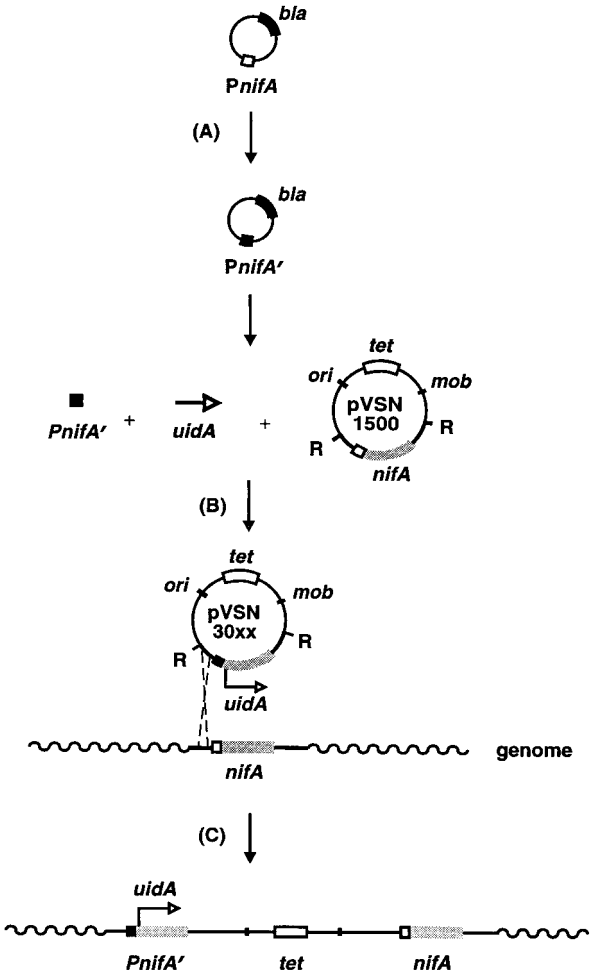


FIG. 2. Mutational analysis of the *Azorhizobium nifA* promoter region. (A) In vitro mutagenesis of the *nifA* promoter; (B) construction of pVSN30xx, a transmissible suicide vector carrying the *nifA* locus and *nifA* promoter variants fused to *uidA*; (C) introduction of pVSN30xx derivatives at the homologous genomic *nifA* locus to yield P*nifA*::*uidA* merodiploids. Abbreviations: *bla*, β-lactamase; *tet*, tetracycline resistance region; *mob*, mobilization site; *ori*, ColE1 replication origin.

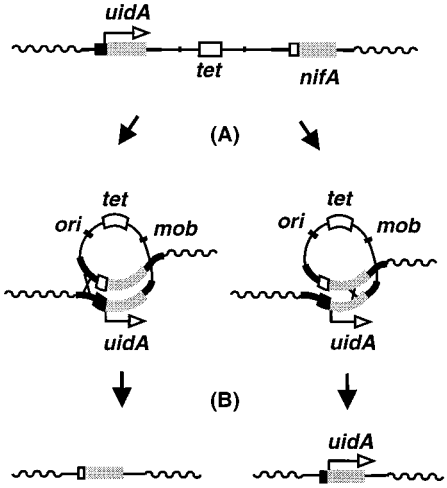


FIG. 3. Perfect gene replacement at the *Azorhizobium nifA* locus. (A) The merodiploid precursor is first cultured in the absence of tetracycline for several generations. The integrated plasmid vector may be excised by a single inverse homologous recombination event anywhere along the duplicated DNA region. (B) Tc^s derivatives represent either wild-type revertants (left) or P*nifA*::*uidA* haploids (right) which constitute perfect gene replacements.

fixJ strain 57592, and *ntrC* strain 571C6 (Table 1). P*nifA* induction patterns were again determined in response to physiological N limitation and/or O_2 limitation (Table 3). Notably, residual P*nifA* activities in both *fixK601* and *ntrC6* backgrounds correlated well with leaky Nif$^-$ phenotypes (28, 43). In the *fixK601* background, P*nifA* induction was quite weak. β-Gus reporter activities were very low (<7%) under all physiological conditions tested, even far below those observed in uninduced cultures of the otherwise wild-type strain 60301. Thus, FIXK serves as a strong *trans*-activator for *nifA* transcription. In the *fixJ592* background, slightly higher β-Gus activities were observed in response to either N limitation (19%), O_2 limitation (11%), or both N and O_2 limitation (21%). Thus, while FIXJ *trans*-activates *fixK* gene expression (28), the *fixJ592* mutant does not fully abolish FIXK activity, as assayed at P*nifA*.

To study the role of the *trans*-activator NTRC, which responds to changes in anabolic N levels, strain 571C6, which carries the *ntrC6*::Tn*5* allele, was studied. In the *ntrC6* background, P*nifA* failed to induce in response to N limitation (4%) but normally responded to the other partially inducing condition, O_2 limitation alone (20%). However, in simultaneous N limitation and O_2 limitation, complete P*nifA* induction was weak (23%). While it weakly *trans*-activates P*nifA*, as is evident in both *fixK* and *fixJ* backgrounds, NTRC is nevertheless required for integrated, high-level P*nifA* activity. This behavior is consistent with interactive P*nifA* regulation.

***Azorhizobium* σ^{54} initiators are encoded by a multigene family, among which *rpoF* and *rpoN* are functionally distinct.** *Azorhizobium* strains 60002 and 60064, generated by VP2021 insertion mutagenesis, were isolated as tight Nif$^-$ mutants. VP2021 insertions confer a Tc^r Km^r phenotype; physically, VP2021 insertion mutants carry triplicate IS*50* elements (16). Recombinant (Vi) plasmids from strains 60002 and 60064 were obtained by direct Vi cloning (16); of necessity, pVi60002 and pVi60064 carried genomic sequences flanking IS*50* insertion points in the respective mutants. Plasmid pVi60064 encoded both tetracycline and ampicillin resistance determinants; as was expected, the VP2021 insertion in strain 60064 resulted from IS*50*R-mediated transposition. In contrast, plasmid pVi60002 encoded both tetracycline and kanamycin resistance determinants; as deduced, the VP2021 insertion in strain 60002 resulted from IS*50*L-mediated transposition (16). Both plasmids were then used to probe a wild-type *Azorhizobium* genomic library constructed in λEMBL3 (15). Three recombinant phages, λNif144, λNif15, and λNif65, were isolated, and insert DNA from each phage was physically mapped with several restriction endonucleases. The three phages carried overlapping homologous *Azorhizobium* DNA, subsequently denoted as the *rpoF* locus (Fig. 4). From physical mapping experiments, both strains 60002 and 60064 carried single, independent *rpoF*::VP2021 insertions that physically mapped some 20 bp apart; the *rpoF* locus is unlinked to *nifA* (Fig. 1 and 4).

In VP2021 insertion mutants, because the two outside IS*50* elements, which delimit insertions, are direct repeats, they serve as a template for homologous recombination. As a consequence, complex VP2021 mutants resolve into simple, allelic IS*50* insertion derivatives from which VP2021 sequences have been precisely excised. Toward this end, strains 60002 and 60064 were cultured in the absence of antibiotics, plated, and screened for Tc^s colonies (Fig. 3) (9). Representative Tc^s de-

TABLE 2. *Azorhizobium* P*nifA* activity in response to physiological O_2 and N status

Genotype	β-Gus activity in response to:							
	↑ O_2 ↑ N		↓ O_2 ↑ N		↑ O_2 ↓ N		↓ O_2 ↓ N	
	Amt[a]	%	Amt	%	Amt	%	Amt	%
60301 P*nifA*::*uidA nifA*$^+$	31	13	57	23	59	24	243	100
60314 P*nifA*::*uidA* (haploid)	18	7	28	12	14	6	381	157
61071 P*nifA*::*uidA nifA107*R	13	5	65	27	55	23	322	133
60341 P*nifA*::*uidA nifA34*R	12	5	23	9	17	7	314	129

[a] Measured in nanomoles per minute per milligram of protein.

rivatives were then analyzed by DNA hybridization experiments; all carried simple *rpoF*::IS*50* alleles (Fig. 4). Two excision derivatives, 60002L, carrying *rpoF2*::IS*50*L (*rpoF2*L) and 60064R *rpoF64*::IS*50*R (*rpoF64*R), were identified and studied further. To construct *rpoF* merodiploids, a 13.2-kbp *Sal*I fragment from λNif15 was subcloned into pSUP2024AA to yield pVSN4000, which was then cointegrated with the *rpoF2*L and *rpoF64*R mutants. Representative Tc^s derivatives from each parent were analyzed; all retained the tight Nif$^-$ parental phenotype (Table 1). In complementation tests, *rpoF64*R *rpoF*$^+$ merodiploids yielded a Nif$^+$ phenotype, whereas *rpoF2*L *rpoF*$^+$ merodiploids remained Nif$^-$. In a series of batch cultures, *rpoF2*L merodiploids were repeatedly selected for N_2-dependent growth; no Nif$^+$ isolates were obtained. Presumably, *rpoF2*L derivatives carry an active IS*50*L promoter (49, 50), whose transcriptional orientation is opposite that of *rpoF* itself (Fig. 4). Thus, the Nif$^-$ phenotype of the *rpoF2*L *rpoF*$^+$ merodiploids might be due to dominant antisense effects. Alternatively, strain 60002L may carry an additional mutation(s).

The *rpoF2*L and *rpoF64*R mutants were then screened for Ntr phenotypes by growth tests on defined media with either succinate, L-malate, or fumarate as the C source and either

TABLE 3. Effects of both *cis*-acting and *trans*-acting mutations on P*nifA* activity in P*nifA*::*uidA nifA* merodiploids

trans-acting mutant background	*cis*-acting mutant(s)	β-Gus activity in response to:							
		↑ O_2 ↑ N		↓ O_2 ↑ N		↑ O_2 ↓ N		↓ O_2 ↓ N	
		Amt[a]	%	Amt	%	Amt	%	Amt	%
Wild type	Wild type	31	13	57	23	59	24	242	100
	NIFAbox′	39	16	54	22	62	26	203	84
	σ^{54}box′	51	21	259	107	62	26	385	159
	Anaerobox′	17	7	17	7	11	5	21	9
	NIFAbox′ σ^{54}box′	26	11	170	70	73	30	273	113
	NIFAbox′ anaerobox′	15	6	9	4	12	5	14	6
	σ^{54}box′ anaerobox′	16	7	14	6	18	7	19	8
	NIFAbox′ σ^{54}box′ anaerobox′	13	5	3	1	2	1	2	1
*nifA107*R NifA$^-$	Wild type	13	5	65	27	55	23	322	133
	NIFAbox′	28	11	50	21	41	17	279	115
	σ^{54}box′	21	9	247	102	58	24	432	178
	Anaerobox′	13	5	26	11	12	5	17	7
	NIFAbox′ σ^{54}box′	26	11	63	26	16	7	46	19
*nifA34*R NifA$^-$	Wild type	12	5	23	9	17	7	314	129
	NIFAbox′	12	5	22	9	19	8	264	109
	σ^{54}box′	17	7	178	73	23	9	412	170
	NIFAbox′ σ^{54}box′	12	5	62	26	20	8	39	16
fixK601	Wild type	5	2	10	4	18	7	18	7
	NIFAbox′	3	1	11	5	18	7	19	8
	σ^{54}box′	20	8	16	7	27	11	26	11
fixJ592	Wild type	9	4	26	11	47	19	52	21
	NIFAbox′	11	4	12	5	27	11	25	10
	σ^{54}box′	11	4	17	7	30	13	36	15
rpoF64	Wild type	5	2	31	13	15	6	77	32
	NIFAbox′	7	3	7	3	13	6	31	13
	σ^{54}box′	6	2	267	110	32	13	489	202
	NIFAbox′ σ^{54}box′	16	7	41	17	16	7	93	38
rpoF2	Wild type	5	2	14	6	9	4	59	24
	NIFAbox′	8	3	17	7	11	5	40	17
	σ^{54}box′	13	5	114	47	19	8	127	52
	NIFAbox′ σ^{54}box′	13	5	25	10	11	5	41	17
ntrC6	Wild type	21	9	48	20	10	4	56	23
	NIFAbox′	27	11	18	7	23	10	44	18
	σ^{54}box′	34	14	94	39	37	15	457	188
	NIFAbox′ σ^{54}box′	15	6	15	6	16	7	97	40

[a] Measured in nanomoles per minute per milligram of protein.

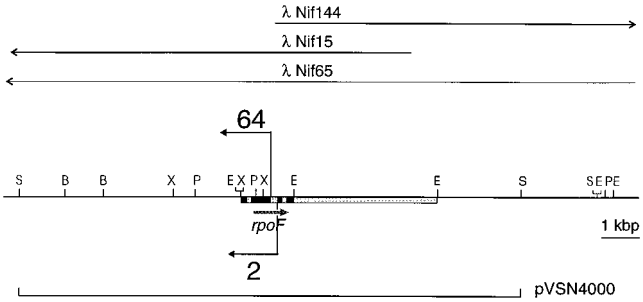


FIG. 4. Map of the *Azorhizobium rpoF* locus. Insertion points for *rpoF* mutants 60002 (labeled 2) and 60064 (labeled 64) are indicated; to convey independent insertion sites, diagrammed spacing between genomic insertion points for the two mutants is exaggerated. Mutant 60002 carries an *rpoF*::IS*50*L insertion; mutant 60064 carries an *rpoF*::IS*50*R insertion (see text). Insert genomic DNA fragments for recombinant library phages λNif144, λNif15, and λNif65 along with plasmid pVSN4000 (Table 1) carrying a subcloned DNA fragment are indicated. Abbreviations: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I.

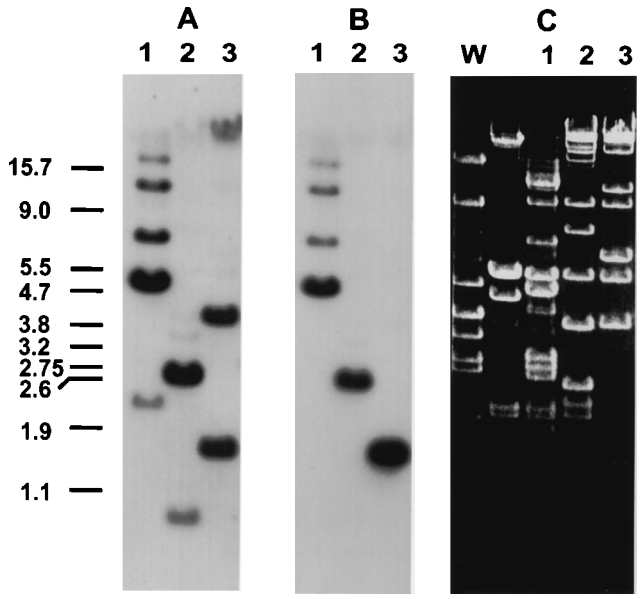


FIG. 5. Hybridization of *Azorhizobium rpoF* locus DNA to the *Bradyrhizobium rpoN*$_2$ probe. Purified recombinant λNif65 DNA was doubly digested with both endonucleases. (A and B) Lanes: 1, *Sal*I and *Pst*I (note that incomplete digestion products are evident); 2, *Sal*I and *Xho*I; 3, *Sal*I and *Eco*RI. Hybridization probes used were *rpoN*$_2$ insert DNA (A) and pVi60002L, the directly cloned product of mutant 60002::Vi2021 (B) (see Fig. 4) (17). (C) Agarose electropherogram used for these Southern transfers stained with ethidium bromide. Lanes: W and the unlabeled lane, molecular size standards; 1, 2, and 3, lanes corresponding to the three lanes of panels A and B. Molecular size standards (in kilobase pairs) are reiterated to the left of the figure.

nitrate, L-leucine, or L-valine as the N source (see Materials and Methods). With the exception of slightly impaired growth on nitrate (opalescent colonies), no differences were observed between the *rpoF* mutant and wild type. In contrast, *ntrC* mutant strain 571C6 did not grow on either nitrate, L-leucine, or L-valine as the N source. Therefore, both *rpoF2*L and *rpoF64*R mutants showed tight Ntr$^+$ Nif$^-$ phenotypes.

σ^{54}F is required for N$_2$ fixation. The *rpoN* gene is functionally conserved among diverse bacteria; *rpoN* encodes σ^{54}, an RNA polymerase initiator subunit, which alters RNA polymerase specificity and which targets promoters carrying unique, highly conserved −12 and −24 consensus sequences (5, 32). In *B. japonicum*, two *rpoN* genes have been identified; both *rpoN*$_1$ and *rpoN*$_2$ single mutants fix N$_2$ at wild-type rates during symbiosis. Thus, both genes are active, and the gene products are functionally degenerate (30). In *Azorhizobium*, an *rpoN* gene has also been identified and sequenced; an *rpoN* insertion mutant shows a Nif$^-$ Ntr$^-$ phenotype, but *nifA* expression is unaffected (56). Therefore, we undertook the isolation of *Azorhizobium rpoN* homologs by genomic hybridization experiments. When λNif15 *Azorhizobium* insert DNA was isolated and probed with *Bradyrhizobium rpoN*$_1$ and *rpoN*$_2$ coding sequences, strong homology was detected with both probes (Fig. 5). To physically map this locus in more detail, *Azorhizobium* DNA was isolated from pVi60002 and pVi60064 and subcloned into pBluescript SK plasmids, and a DNA fragment carrying *rpoF* (Fig. 4) was sequenced. The *rpoF* coding sequence proved different from that of *Azorhizobium rpoN* (56) but ~90% homologous to both *Bradyrhizobium rpoN* genes; thus, *Azorhizobium rpoF* also encodes a σ^{54} initiator (34a).

In *Azorhizobium* genomic hybridization experiments with either *Bradyrhizobium rpoN*$_1$ or *rpoN*$_2$ as a probe, yet another *rpoN* homolog distinct from both *Azorhizobium rpoN* and *rpoF* was identified; mutations in this gene were not obtained (33). Therefore, *Azorhizobium* encodes at least three, different σ^{54} initiators, and the two studied are functionally distinct. Among diverse bacteria, including rhizobia and enteric bacteria, bona fide *rpoN* mutants show a tight Ntr$^-$ phenotype. Because *Azorhizobium rpoF2*L and *rpoF64*R mutants both show an Ntr$^+$ phenotype, this locus was given its distinct name.

σ^{54}F is also required for high-level P*nifA* activity. To allow reporting of P*nifA* activities, strains 60002L and 60064R, carrying distinct *rpoF*::IS*50* alleles, were next converted to P*nifA*::*uidA nifA*$^+$ merodiploids, as described above. When the resulting *nifA* merodiploid RpoF$^-$ derivatives 60302 and 60364 (Table 1) were cultured under simultaneous N and O$_2$ limitation, P*nifA* was partially induced (32 and 24%, respectively). However, for both derivatives, P*nifA* induction was very low in response to N limitation alone (6 and 4%, respectively). Likewise, when cultured in excess N under air, P*nifA* levels were drastically reduced (2%). Only in strain 60364 was there some P*nifA* induction in response to O$_2$ limitation alone (13%), which was still weak. Whereas mutations in other P*nifA trans*-activators showed leaky Nif$^-$ phenotypes, both *rpoF2*L and *rpoF64*R single mutants yielded tight Nif$^-$ phenotypes in growth tests and showed <1% wild-type dinitrogenase activities. The *rpoF64*R mutation presumably confers a leaky NifA$^-$ phenotype. To reconcile the tight Nif$^-$ phenotype of *rpoF* mutants, σ^{54}F might be absolutely required for expression of downstream *nif* genes, whose expression is also NIFA dependent.

Analysis of *Azorhizobium* P*nifA cis*-acting elements. To analyze *cis*-acting P*nifA* elements, tandem dinucleotide changes were introduced at each of three putative elements, the anaerobox, the NIFAbox, and the σ^{54}box, on plasmid templates. All mutations were verified by DNA sequencing. DNA fragments carrying these mutations were then spliced in vitro into the P*nifA*::*uidA* reporter gene of plasmid pVSN3001 (Table 1). Seven distinct pVSN30xx plasmids, which carried combinatorial P*nifA* mutations in *cis* to the *nifA*::*uidA* reporter, were obtained (see Materials and Methods). Next, wild-type P*nifA* and the combinatorial (P′*nifA*) mutants were introduced into eight different *Azorhizobium* backgrounds, i.e., wild type, *nifA107*R, *nifA34*R, *ntrC6*, *fixK601*, *fixJ592*, *rpoF2*L, and *rpoF64*R. The resulting *nifA* merodiploids were cultured in either O$_2$ limitation, N limitation, or simultaneous O$_2$ and N limitation. To normalize P*nifA* activities in all subsequent ex-

TABLE 4. Sequence comparison of *Azorhizobium cis*-acting box elements

Gene	Sequence
NIFAbox elements	
nifH$_1$	**G**GAAGACG**TGT**TTCC**T**TTCCA**ACA**
nifH$_1$	**G**TGATCTC**GTC**CTCG**T**CCACG**ACA**
nifH$_2$	**A**CCAGCGA**TGT**CGCG**T**TTGAA**ACA**
nifH$_2$	**T**TTTACTT**G**A**T**TAAT**T**CGACC**ACA**
nifA	**G**TCTCCGC**GGT**TTCCATTATA**ACA**
fixO	GAGC**TC**GGCC**T**CTATG**ACA**
fixA	**T**GCGCGTT**TTC**GGGC**T**GAAAG**ACA**
σ^{54}box elements	
nifA	A**GGAGGCT**G**A**TCC**C**T**CGCA**
nifH$_1$	AT**GGGG**A**T**AT**G**AA**G**A**CGCA**
nifH$_1$	T**G**A**GGGCTGAG**T**ACACGCA**
nifO	GC**G**C**GG**TGA**A**CGT**C**G**CGCA**
nifA	A**G**CC**GG**GGCG**G**A**A**AG**CGCA**
nifH$_2$	CACT**GGC**AC**A**CCCGTT**GCA**

periments, β-Gus activities were measured at three time points during rapid induction, and P*nifA* activities were calculated as relative rates multiplied by fully induced levels in the wild type (Table 3).

At P*nifA*, high-level activity requires the anaerobox element in *cis*. A 14-bp anaerobox element with dyad symmetry lies 131 bp upstream of the P*nifA* σ^{54}-dependent transcription start (40, 46). The anaerobox is highly conserved among genes transcriptionally regulated by both *E. coli* FNR, which activates genes expressed under low O_2 (55), and FIXK, an FNR ortholog, which is highly conserved among the rhizobia, including *Azorhizobium* (1, 3, 28). At their C termini, both FNR and FIXK carry absolutely conserved helix-turn-helix motifs (4), which, by analogy to *E. coli* cyclic AMP-binding protein, the archetype for this family (11), should mediate specific DNA binding to anaerobox elements. Accordingly, a mutant P[anaerobox]*nifA* element was constructed in *cis* to the P*nifA*::*uidA* reporter gene as a tandem TT→GG transversion (*nifA* sequence positions −130 and −131 relative to the strong, σ^{54}-dependent transcription start point) corresponding to the first and second anaerobox nucleotide pairs (Fig. 1). The resulting P[anaerobox′]*nifA*::*uidA* reporter was introduced into an otherwise wild-type background. When cultured under all test conditions, the P[anaerobox′]*nifA*::*uidA* *nifA*$^+$ merodiploid showed low P*nifA* activity, at best only 9% of fully induced wild-type levels, compared with 2% of fully induced levels for wild-type P*nifA* in the *fixK*601 background (Table 3). Compared with other *cis*- and *trans*-acting elements, P*nifA* induction shows the greatest dependence on the anaerobox element in *cis* and FIXK in *trans*. This result is consistent with, but does not constitute direct evidence for, operation of a FIXK-anaerobox DNA complex at P*nifA*. Recall that when cultured under simultaneous N excess and O_2 excess, the uninducing condition, otherwise wild-type *nifA*::*uidA* *nifA*$^+$ merodiploids showed 13% induced P*nifA* activity. Because P[anaerobox′]*nifA* mutants never achieved even these low levels, uninduced wild-type P*nifA* activity might reflect partial intracellular O_2 limitation, even in vigorously aerated, exponential-phase cultures.

P*nifA* activity is only marginally affected by NIFAbox element mutations. Located 102 bp upstream from the σ^{54}-dependent transcription start, P*nifA* carries a sequence closely resembling a consensus NIFA DNA-binding site (NIFAbox) (37) found upstream of *nif* operons in diverse, diazotrophic bacteria (23) (Table 4). With similar methodology, a tandem AC→GT double-transition mutation was introduced in this NIFAbox element at *nifA* positions −83 and −84 in *cis* to the P*nifA*::*uidA* fusion reporter (Fig. 1). *Azorhizobium nifA* merodiploids carrying this P[NIFAbox′]*nifA*::*uidA* reporter allele were then isolated. In the *rpoF64*R background, the P[NIFAbox′]*nifA* mutant showed no partial induction in response to O_2 limitation, as had been observed for wild-type P*nifA* (Table 3). In all other genetic backgrounds, including *nifA* coding sequence mutants *nifA34*R and *nifA107*R (Fig. 1B), P[NIFAbox′]*nifA* allowed partial induction in response to either limiting N or limiting O_2. Yet, consistently, P[NIFAbox′]*nifA* failed to allow full induction at wild-type levels in response to simultaneous N and O_2 limitation (Table 3). Recall that wild-type P*nifA* showed hyperactivity in the absence of NIFA in *trans* (Table 2). If, indeed, the mutant NIFAbox′ element eliminated NIFA binding at this site, then either P*nifA* responds differently to the absence of NIFA in *cis* versus in *trans* or, alternatively, the P[NIFAbox′]*nifA* mutation more than simply eliminates NIFA binding at this site. As discussed below, the latter seems true.

The σ^{54}box, which correctly positions P*nifA* transcription initiation, acts both positively and negatively. Immediately upstream of the σ^{54}-dependent transcription start lies a 16-bp sequence conforming to a σ^{54}box element, which is diagnostic of σ^{54}-type promoters (5). Indeed, purified *Klebsiella* σ^{54} strongly binds σ^{54}box element DNA in vitro (7). As with anaerobox′ and NIFAbox′ mutants, a σ^{54}box GG→TT double-transversion (P[σ^{54}box′]*nifA*) was constructed at *nifA* sequence positions −25 and −26 and on a plasmid P*nifA*::*uidA* template (Fig. 1). Similarly, *Azorhizobium nifA* merodiploids carrying the P[σ^{54}box′]*nifA*::*uidA* reporter were then selected and studied. Surprisingly, the P[σ^{54}box′]*nifA* mutant allowed hyperactivity (Table 3). For example, in the wild-type background, P[σ^{54}box′]*nifA* allowed 159% (of wild-type P*nifA*) induction in response to simultaneous N and O_2 limitation. Hyperactivity was even greater in the presence of *nifA* coding sequence mutant *nifA34*R and *nifA107*R (178 and 170%, respectively), *ntrC6* mutant (188%), and *rpoF2*L and *rpoF64*R mutant (202 and 52%, respectively) backgrounds. In response to O_2 limitation alone, P[σ^{54}box′]*nifA* was fivefold stimulated in the wild-type background, a much stronger response when compared with that of the P*nifA* wild type, whereas in response to N limitation, induction was only 20% of that observed with the P*nifA* wild type. Indeed, in both *fixK601* and *fixJ592* mutant backgrounds, P[σ^{54}box′]*nifA* allowed no response to O_2 limitation alone, and in the *fixK* background, P[σ^{54}box′]*nifA* allowed no response to simultaneous N and O_2 limitation. Thus, both the P[σ^{54}box] element in *cis* and NIFA in *trans* have positive and negative controlling activities at P*nifA*.

Multiple P*nifA* *cis*-acting mutations—NIFA, in *trans*, targets both the NIFAbox and the σ^{54} box for autoregulation. With the methodology discussed above, *Azorhizobium nifA* merodiploids carrying double- and triple-mutant P[anaerobox′]*nifA*, P[NIFAbox′]*nifA*, and P[σ^{54}box′]*nifA* elements in all combinations were constructed in *cis* at the *nifA*::*uidA* plasmid reporter. Resulting plasmids were then used to construct *nifA* merodiploid derivatives carrying combinatorial *cis*-acting mutations. In an otherwise wild-type background, the effects of the three *cis*-acting mutations on P*nifA* activity were essentially additive. When combinatorial mutants carried the P[anaerobox′] element, P*nifA* activity was negligible (1 to 8% of fully induced activity) under all physiological conditions (Table 3). Thus, the anaerobox is required in *cis* for high-level activity of P*nifA* under all conditions. Because the P[anaerobox′] element phenotype was epistatic to phenotypes conferred by the P[NIFAbox′]*nifA* and P[σ^{54}box′]*nifA* mutations, its effects were considered only in wild-type and NIFA backgrounds. The

P[NIFAbox′, σ^{54}box′]*nifA* double mutant allowed some hyperactivity (70% under O_2 limitation; 113% in response to simultaneous O_2 and N limitation), which was still below levels observed with the P[σ^{54}box′]*nifA* single mutant. However, in the *nifA107*R and *nifA34*R backgrounds, the P[NIFAbox′, σ^{54}box′]*nifA* double mutant was poorly induced (19% under O_2 limitation; 16% in response to simultaneous O_2 and N limitation), whereas the parental single-promoter mutants were more strongly induced. Thus, in the absence of NIFA, P[σ^{54}box′]*nifA* hyperactivity required a wild-type NIFAbox element. These results and the earlier analysis of P[NIFAbox′]*nifA* single mutants both implicated a second upstream transcription start located at, and superimposed upon, the NIFAbox element.

At P*nifA*, NIFA binding masks an upstream transcription start(s). Recall that both NIFA in *trans* and the σ^{54}box element in *cis* negatively as well as positively regulate P*nifA* activity and that *rpoF* mutants retained P*nifA* induction in response to O_2 (Table 3). Such results were difficult to reconcile with simple, single-transcript models. Might P*nifA* initiate multiple mRNAs? To test this possibility, *nifA* mRNA 5′ start point(s) was mapped with an in vitro primer extension assay (see Materials and Methods). For obvious reasons, while we preferred to carry out these experiments with haploid *nifA* strains, we were unable to convert either strain 60341-3030, carrying the P[σ^{54}box′]*nifA*::*uidA nif34*R merodiploid, or strain 61071-3030, carrying the P[σ^{54}box′]*nifA*::*uidA nifA107*R merodiploid, to haploid P[σ^{54}box′]*nifA* mutant derivatives. Therefore, *nifA* merodiploids were included in start point analyses. Because the P[NIFAbox′]*nifA* double-transition mutation might also have compromised an upstream start(s), leading to ambiguous physiological behavior, such mutants were excluded from start point analysis. Total RNA was purified from fully induced N_2-fixing cultures of five strains, namely, 57100 (wild type), 60107R (*nifA107*R), 60064R (*rpoF64*R), 60301-3030 (P[σ^{54}box′]*nifA*::*uidA nifA*$^+$), and 61071-3030 (P[σ^{54}box′]*nifA*::*uidA nifA107*R). For the former four strains, a strong primer extension product whose template 5′ end corresponded to *nifA* nucleotide position +1 was observed. Because it is 12 bp distal to the σ^{54}box element (Fig. 1), position +1 likely represents a strong transcription start. Besides the transcript representing the +1 start point, within the size range of interest, longer primer extension products were not observed for any of these four strains (Fig. 6). For mRNA isolated from strain 61071-3030, three additional 5′ start points, at positions −82,−74, and +26, were mapped (Fig. 6). The two upstream 5′ start points are, respectively, 36 and 44 bp distal to the anaerobox element (Fig. 1). For both FNR- and FIXK-dependent transcripts in diverse bacteria, including enteric organisms, such a spacing between bound *trans*-activator and the 5′ start point is highly conserved (40). Notably, the sequences TTGTCT-N_{13}-TATAAC (or alternatively, TTGTCT-N_{16}-AACAAT) are related to consensus, enteric bacterial, σ^{70}-dependent transcription initiation sites. If these sequences constitute functional σ^{70}-type box homologs, they might direct upstream transcription initiation (Fig. 1). Indeed, one observed upstream start point maps 10 bp distal to the inferred σ^{70}-type box and is thus correctly positioned to allow FIXK-dependent activation of RNA polymerase housekeeping-type initiation complexes at that site (Fig. 1). Since the NIFAbox is superimposed over the putative σ^{70}-type element(s), any bound NIFA might preclude RNA polymerase housekeeping-type initiation complex formation.

Primer extension reactions with retroviral reverse transcriptase as the catalyst and purified *Azorhizobium* mRNA (68% G+C content) as the template are problematical and result in frequent abortive terminations (Fig. 6). Because the analyzed P*nifA* (−82 to +55) region has a 65% G+C content, relatively stable mRNA secondary structures might also exacerbate premature chain termination during reverse transcription. Hence, these primer extension results are qualitative and do not allow quantitative inferences about *nifA* mRNA initia-

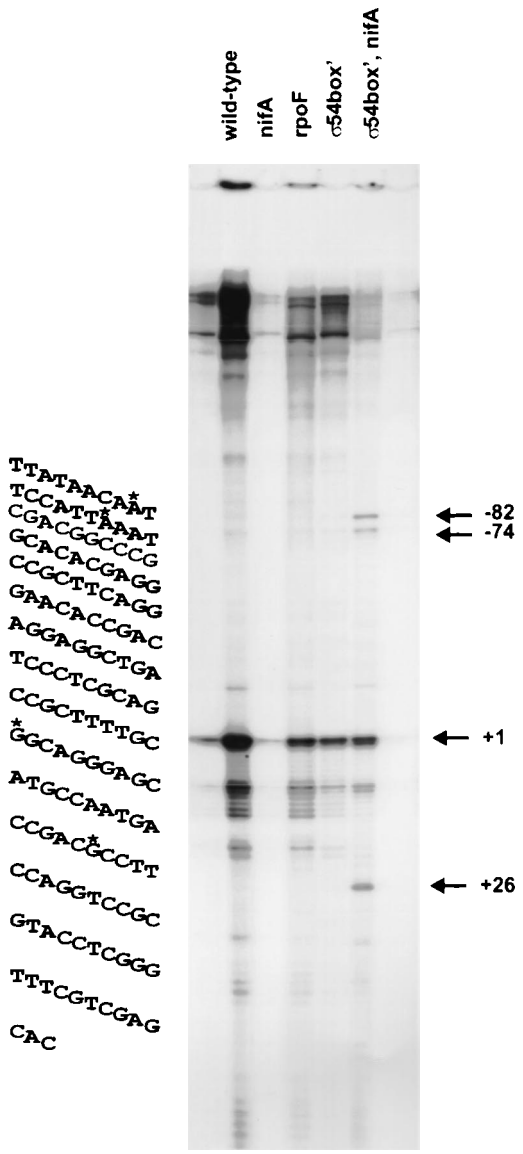


FIG. 6. Transcription start point mapping of *nifA* mRNAs. For five strains, total RNA was purified from N_2-fixing cultures of the following five strains: 57100 (wild type), 60107R (*nifA107*R), 60064R (*rpoF64*R), 60301-3030 (P[σ^{54}box′]*nifA*::*uidA nifA*$^+$), and 61071-3030 (P[σ^{54}box′]*nifA*::*uidA nifA107*R). Samples of purified RNAs were annealed with a synthetic radiolabeled oligonucleotide primer (3′-AGCTCGTGGCGGCCTCTGTA-5′) corresponding to *nifA* +57 to +76 coding sequence (Fig. 1) and primer extended with purified retroviral reverse transcriptase in the presence of the four deoxynucleoside triphosphates. The same synthetic deoxynucleotide was used as the primer in standard dideoxynucleotide chain termination DNA sequencing reactions with the 4.9-kbp *Eco*RI (*nifA*$^+$) DNA fragment as the template. RNA primer extension reactions and DNA sequencing reactions were loaded on adjacent lanes of denaturing polyacrylamide gels for DNA sequencing and subjected to electrophoresis and autoradiography. For ease of interpretation, the inferred DNA sequence is substituted for sequencing lanes, as shown to the left of the figure. Nucleotide bases in these sequences capped with asterisks denote inferred *nifA* 5′ start points (see text).

tion frequencies at multiple 5′ start points. Both *nifA* merodiploid strains 60301-3030 and 61071-3031 showed +1 start point activity, presumably owing to the wild-type σ^{54}box copy (Fig. 6). Because upstream transcripts were observed only in *nifA* merodiploid strain 61071-3030, $NIFA_{107R}$ somehow interferes with negative regulation by $NIFA_{WT}$ at P*nifA*. Furthermore, because upstream start points went undetected in both strains 60107R and 60301-3030, efficient upstream initiation requires knockout both of $NIFA_{WT}$ repression and of the *cis*-acting σ^{54}box element. The observed P*nifA* hyperactivity in these mutants presumably reflects increased upstream transcription initiation rates.

DISCUSSION

Previously, from promoter region sequence comparisons and transcript mapping studies, regulation of *Azorhizobium nifA* gene activity was recognized as potentially complex (40, 46, 56). As documented here, the *Azorhizobium nifA* gene carries two promoters, an upstream FIXK-regulated σ^{70}-type promoter that is superimposed on a downstream NTR-regulated σ^{54}-type promoter. The start points for the σ^{54}-type promoter lie some 80 bp apart. The upstream σ^{70}-type promoter assignment is made by analogy with enteric and other bacteria; an *Azorhizobium* σ^{70} homolog has been neither identified nor studied. In support of this assignment, the hypothetical σ^{70}-type box maps 10 bp distal to the mapped upstream promoter. As studied in other bacteria, activities of σ^{70}-type and σ^{54}-type promoters are normally independent. At *Azorhizobium nifA*, however, the two promoter activities are interdependent. Formally, the upstream σ^{70}-type promoter positively regulates the downstream σ^{54}-type promoter, and the downstream promoter negatively regulates the upstream promoter. In terms of transcript yield, the upstream σ^{70}-type promoter is then effectively weak, and the downstream σ^{54}-type promoter is strong. mRNA mapping of coding sequence transcripts supports these assignments. Notably, such designations of promoters as operatively weak or strong might inaccurately represent in vivo initiation rates. Nascent RNAs which terminated upstream of the oligonucleotide primer would go unreported in these transcript mapping experiments. Moreover, for interactive promoters, weak and strong assignments may vary with genetic background. Indeed, P*nifA* hyperactivity observed for both σ^{54}box (*cis*-acting) mutants and *nifA* (*trans*-acting) mutants might represent predominantly upstream σ^{70}-type promoter activity, which is then strong.

At the *Bradyrhizobium fixR nifA* promoter, σ^{70}-type and σ^{54}-type promoters are also juxtaposed, but the overlap is complete; transcription start points map only 2 bp apart. Regulation of overlaid *Bradyrhizobium* promoters may reflect direct competition for DNA binding among RNA polymerase σ^{70}-type and σ^{54}-type complexes (3).

Among three *trans*-activators, FIXK, NIFA, and NTRC, which together regulate *Azorhizobium* P*nifA* activity, FIXK is strong. Conceivably FIXK, in response to limiting O_2, might activate P*nifA* in both direct and indirect modes. Directly, FIXK, bound at the anaerobox, might activate transcription initiation at the upstream σ^{70}-type start point(s). Indirectly, this σ^{70}-type upstream promoter activity somehow stimulates transcription initiation at the downstream σ^{54}-type promoter. In response to physiological N limitation, both NTRC and NIFA might directly activate the downstream σ^{54}-type promoter. At P*nifA*, however, while NIFA is only a weak activator, it is a strong repressor. If bound to the NIFAbox, NIFA might repress and/or inhibit (i) FIXK activation of the upstream σ^{70}-type promoter, (ii) NTRC activation of the downstream σ^{54}-type promoter, and (iii) FIXK (indirect) activation of the downstream σ^{54}-type promoter. These inferences rest on the presumption that FIXK binds the anaerobox element and that NIFA binds the NIFAbox element in vivo (37). These activities have not been reconstructed in vitro. Despite extensive work with various bacteria, neither active wild-type FIXK nor active wild-type NIFA has been purified from any microorganism. From sequence analyses of diagnostic, conserved cysteine residue motifs, both FIXK and NIFA probably carry redox-active iron-sulfur centers, which would complicate their purification in active form.

From DNA sequence analysis, no canonical NTRCbox element is evident in the 2.0-kbp interval spanning P*nifA* (40). Hence, at P*nifA* in vivo, NTRC is dilute and its activation is weak. While a putative NIFAbox element able to bind NIFA is present at P*nifA*, its activity is probably weak. The P*nifA* NIFAbox contains a GGT sequence in lieu of the canonical TGT and thus lacks the expected dyad symmetry. In contrast, the unlinked *Azorhizobium* $nifH_1$ and $nifH_2$ promoters both carry tandem NIFAbox elements; for each *nifH* promoter, one NIFAbox is canonical, and the other is less so (Table 4). As a general feature, promoters for autoregulatory genes show low-affinity last-to-fill *cis*-acting binding sites, which, when filled, effectively cap transcription rates and establish ceilings for induced, steady-state levels of such autoregulators (23).

NIFA is a member of the response-regulator family for which NTRC serves as the archetype (19). NTRC binds specific DNA sites usually located upstream of target operons (47). From comparative analyses of their modular structures, both NTRC and NIFA carry homologous central domains, which recognize RNA polymerase σ^{54}, and diagnostic C-terminal helix-turn-helix motifs, which specifically bind NTRCbox or NIFAbox DNA elements, respectively. However, NTRC and NIFA possess quite heterologous N-terminal domains which span ~200 residues and thus are presumably functionally important (18, 36). In the case of NTRC, a conserved aspartate residue is phosphoacylated by NTRB, its cognate histidine kinase; thus, the NTRC N terminus is a bona fide response domain (58). When phosphoacylated, NTRC facilitates RNA polymerase σ^{54} ternary open complex formation with promoter DNA, which stimulates transcription (45).

Whether the NIFA N terminus also constitutes a response domain and to what it might respond remain uncertain. At *Azorhizobium* P*nifA*, inferred FIXK and NIFA binding sites are adjacent, and notably, FIXK somehow activates the downstream σ^{54}-type promoter. Nevertheless, that FIXK might activate NIFA, which then activates the downstream, σ^{54}-type promoter, is unlikely. Recall that under fully inducing conditions, P*nifA* is hyperactive in the absence of NIFA, and that under partially inducing conditions, in response to limiting O_2, FIXK stimulates P*nifA* even in the absence of NIFA. Moreover, both σ^{54}-type and σ^{70}-type promoters constitute mutually exclusive classes. In well-studied bacteria, NTRC and NIFA target σ^{54}-type promoters; FIXK targets σ^{70}-type promoters. In addition, in *E. coli*, two FIXK homologs, FNR and CRP (cyclic AMP receptor protein), both require specific DNA-binding sites immediately proximal to RNA polymerase σ^{70} initiation complexes. *E. coli* CRP and its homolog FNR both stimulate transcription initiation at short, fixed distances downstream of strong DNA-binding sites (11, 59). In contrast, NTRC and NIFA, much like eukaryotic enhancer-binding proteins, *trans*-activate when DNA bound up to 1 kbp away. Why might CRP, FIXK, and FNR require immediate DNA binding for transcription activation? From a structural analysis of the crystallized complex, CRP bends DNA some 90° upon binding (52), which, obviously, is only a local effect.

In primer extension analyses, reactions yielded laddered reverse transcripts of various deoxynucleotide lengths. Thus, reverse transcription with *Azorhizobium nifA* RNA templates is poorly processive. This may reflect the 68% G+C content of *Azorhizobium* mRNA, the secondary structure at mRNA 5′ ends, or both. Indeed, the product of the *nrfA* gene, which encodes a homolog of *E. coli* HF-1, a small RNA-binding protein, is also required for *nifA* expression (26). Conceivably, NRFA binding of *nifA* mRNAs might facilitate translation. However, NRFA might play an important role in transcription as well. In any case, primer extension experiments yield only qualitative results. As analyzed by electrophoresis, the longest primer extension products, when compared with DNA sequence ladders, presumably map bona fide transcription starts.

In the *fixK* background, P*nifA* showed very little activity under all physiological conditions. Previously, when P*nifA* activities on plasmids were studied, significant basal activities were observed (28). As evidenced here, however, genomic P*nifA* reporter alleles have vanishingly low basal activities. Therefore, real P*nifA* induction requires FIXK and, by inference, physiological O_2 limitation. Because both basal *nifA* transcription and partial induction in response to N limitation were also FIXK dependent, the *Azorhizobium* intracellular environment seems somewhat O_2 limited, even under aerobic batch culture conditions. Indeed, *Azorhizobium* fixes N_2 in aerobic environments (17), and partial FixLJ-dependent activation of *fixK* is evident even in aerobic cultures (27, 28). Therein, limiting intracellular O_2 might be a consequence of high *Azorhizobium* respiratory rates, which reflect simultaneous activities of multiple terminal cytochrome oxidases (29).

In three different *nifA*-coding mutant backgrounds, P*nifA* was hyperactive in response to simultaneous O_2 and N limitation, when compared with the *nifA*$^+$ background. Such negative autoregulation might have physiological relevance at high, steady-state NIFA levels and might serve to maintain homeostasis. For *B. japonicum*, a repressor-like role for NIFA has also been proposed (30). In the *nifA107*R-coding mutant background, partial P*nifA* activation in response to N limitation was similar to that of the wild type, but negative autoregulation was abolished. The IS*50*R insertion in strain 60107R is a distal mutation (15, 40). It is important to recall that the NIFA C terminus encodes its presumed DNA-binding domain. If the *nifA107*R allele produces C-terminally-truncated NIFA, negative autoregulation, which requires NIFA in *cis* (binding to the NIFAbox) might be abolished, but $NIFA_{107R}$ might remain active in *trans*. Moreover, *Klebsiella* NIFA (8, 39) and *Rhizobium* NIFA (2) weakly *trans*-activate in the absence of NIFAbox DNA sequences. Because *Azorhizobium nifH*$_1$*DK* operon transcription is completely abolished in the *nifA107*R mutant (34), any *trans*-activation by $NIFA_{107R}$ must be quite weak.

In all organisms studied to date, identified σ^{54} initiators are functionally equivalent and thus are true orthologs. Except for *B. japonicum* (32), these organisms carry a single *rpoN* gene, which encodes σ^{54}. However, in *Azorhizobium*, multiple genes encode functionally distinct σ^{54} initiators. Like *Bradyrhizobium rpoN*$_2$ mutants, *Azorhizobium rpoF* mutants use dicarboxylic acids (Dct$^+$ phenotype) and secondary N sources (Ntr$^+$ phenotype); only nitrate assimilation is slightly impaired. In contrast, *Klebsiella rpoN* mutants, *Rhizobium rpoN* mutants (48), and an *Azorhizobium rpoN* mutant are all Nif$^-$ and Ntr$^-$ (56). Like *Rhizobium* and *Klebsiella rpoN* mutants, *Azorhizobium rpoF* mutants exhibit a tight Nif$^-$ phenotype and completely fail to express both *nifH*$_1$ and *nifH*$_2$ promoters (34). Thus, both *Azorhizobium* σ^{54}F and σ^{54}N seem required for N_2 fixation. Because *rpoF* mutants retain an Ntr$^+$ phenotype, that σ^{54}F might activate *rpoN* transcription is unlikely.

The physiological significance, if any, of multiple σ^{54} initiators with different promoter specificities remains unclear. Notably, although *rpoF* mutants remain Ntr$^+$, σ^{54}F is required for both NTRC and NIFA *trans*-activation at P*nifA* in response to a limiting anabolic N signal. Multiple σ^{54} initiators might then discriminate among variant σ^{54}box elements but might not discriminate among multiple activators, such as NTRC and NIFA. Indeed, when σ^{54}box element sequences at five *Azorhizobium* σ^{54}-type promoters are compared, all show significant diversity (Table 4). Changes in relative pool sizes of various σ^{54} initiators might also underlie regulation. Hypothetically, for example, a relative increase in the σ^{54}F pool might effectively uncouple N_2 fixation from Ntr regulation, as occurs during symbiotic N_2 fixation. Indeed, in *Bacillus subtilis* sporulation, a well-documented genetically programmed shift in σ initiator pools drives global developmental regulation (35).

In summary, limiting O_2→FixLJK and limiting N→NtrBC signals converge at the *Azorhizobium nifA* gene, target two superimposed promoters, and interactively regulate transcription. In bacteria, such interactive regulation of transcription by two extrinsic signaling pathways seems a relative novelty. Rather, bacterial genes are most frequently regulated by one extrinsic and one intrinsic (to that pathway) signal. Despite the single case at hand, the corollary "one extrinsic signal, one promoter" is a tempting extrapolation. If true, this complicating constraint and the attendant ambiguities of multisignal gene regulation might help to explain the apparent rarity of this phenomenon in bacteria. Among eukaryotes, in which transcription requires variable, complex sets of initiation factors and involves large numbers of *cis*-acting elements which each respond to discrete *trans*-activators, interactive regulation may be the norm.

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